Int. J. Cancer: **100,** 414–418 (2002)
Published 2002 Wiley-Liss, Inc.

Publication of the International Union Against Cancer

HRAS1 VARIABLE NUMBER OF TANDEM REPEATS POLYMORPHISM AND RISK OF BLADDER CANCER

Carla H. VAN GILS[1], Kathleen CONWAY[2], Yu LI[2] and Jack A. TAYLOR[1]*

[1]*Molecular and Genetic Epidemiology Section, Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC, USA*
[2]*Department of Epidemiology, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*

The *HRAS1* variable number of tandem repeats (VNTR) polymorphism, 1 kb downstream from the *HRAS1* gene, has been reported to be associated with risk of various cancers. To examine whether individuals with rare *HRAS1* VNTR alleles are at increased risk of bladder cancer we carried out a case control study with 230 bladder cancer cases and 203 hospital-based controls frequency-matched on ethnicity, gender and age. For genotyping we used a PCR-based long-gel electrophoretic assay that provides precise allele size discrimination. We did not find evidence of a strong overall effect of the *HRAS1* VNTR on bladder cancer risk. Genotype data for whites and blacks were analyzed separately, but the number of black subjects was too small to estimate meaningful odds ratios. Compared to white subjects with 2 common alleles, the odds ratio (OR) for white subjects with 1 rare allele was 0.9 (95% confidence interval (CI) = 0.5–1.4) and for those with 2 rare alleles OR = 1.7 (95% CI = 0.6–5.4). *HRAS1* genotype may be related to the prognosis of bladder cancer, however, because incident cases, *i.e.*, newly diagnosed cases had a higher frequency of rare alleles than did prevalent cases, *i.e.*, cases already existing at the time of recruitment. Repeating the analyses with incident cases only (*n* = 53), the OR for subjects with 1 rare allele was 1.2 (95% CI = 0.6–2.4) and for those with 2 rare alleles 3.2 (95% CI = 0.8–13.7). The number of incident cases was too small to draw firm conclusions on a possible association with a subgroup of tumors with a poor prognosis.
Published 2002 Wiley-Liss, Inc.†

Key words: *bladder cancer; polymorphism;* HRAS1*; variable number tandem repeat (VNTR)*

Rare alleles of the *HRAS1* minisatellite, or variable number of tandem repeat (VNTR), have been associated with risk of various cancers, including bladder cancer.[1] The *HRAS1* VNTR maps 1 kb downstream from the polyadenylation signal of the human proto-oncogene *HRAS1* and is comprised of 30–100 tandemly repeated copies of a 28-base pair (bp) consensus motif.[2] The 4 most common alleles, a1, a2, a3 and a4,[3] have sizes of 1.0 kb, 1.45 kb, 2.05 kb and 2.5 kb, respectively[4] and have a combined allele frequency of 94% in the US population.[1] More than 40 other allelic variants have been described: the 'rare alleles.' Lineage analysis indicates that all rare alleles are derived from the common alleles, usually the one nearest in size.[5] Rare alleles differ from the common alleles in the number of repeats and have slight internal sequence variations in the 28-bp consensus sequence.[6]

Several explanations have been proposed for the relationship between the *HRAS1* VNTR and cancer risk. The *HRAS1* VNTR binds at least 4 members of the rel/NF-κB family of transcriptional regulatory factors,[7] suggesting that the VNTR could affect cancer susceptibility by transcriptional modulation of *HRAS1* or other nearby genes. Interestingly, some allele-specific effects have been observed: the rare a2.1 allele possessed a 2-fold greater enhancer activity than the common a1 and a2 alleles.[8] In lung cancer patients rare *HRAS1* alleles have been shown to be associated with microsatellite instability at several loci,[9] suggesting that rare *HRAS1* alleles may be markers for inherited global genetic instability.[10] Lastly, rare alleles may simply be markers for nearby genes related to cancer risk.

Studies on the relationship between the *HRAS1* VNTR and bladder cancer risk have shown conflicting results.[1,11,12] We present a case-control study that examines the relationship between the *HRAS1* VNTR and bladder cancer risk and also whether the VNTR is related to prognostic characteristics of bladder cancer, such as tumor grade and stage. We employed a PCR-based long-gel electrophoretic assay, which offers a higher resolution over traditional Southern-blot based techniques and therefore a higher sensitivity to detect rare alleles.

MATERIAL AND METHODS

Study population

The study population has been described previously.[13] Briefly, bladder cancer cases (n = 245) and control subjects (n = 215) were enrolled from Urology Clinics at Duke University Medical Center and the University of North Carolina Hospitals. Cases were urology clinic patients with histologically confirmed transitional cell carcinoma of the bladder. Control subjects were urology clinic patients who had no history of any cancer, other than non-melanoma skin cancer. Controls were frequency matched to cases based on ethnicity, gender and age (10-year intervals). The most common diagnoses among controls were benign prostatic hypertrophy and impotence. All individuals were administered a questionnaire that detailed their smoking and other exposure histories. After giving written informed consent, subjects provided blood samples collected under protocols approved by the institutional review boards of each participating institution and these were stored at −80°C until extracted. Analyses of *HRAS1* genotypes were done in 221 cases (90% of eligible cases) and 202 controls (94% of eligible controls).

Genotype analyses

DNA was extracted from peripheral blood lymphocytes by standard phenol-chloroform extraction methods, resuspended in TE buffer (10 mM Tris, 1 mM EDTA) and frozen until used. For *HRAS1* allele length typing, PCR amplification was carried out using 125 ng germline DNA in a 50 ul reaction consisting of 50 mM Tris-HCl, pH 9.2, 14 mM ammonium sulfate, 1.75 mM $MgCl_2$, 300 nM each primer (5′-GCTCCTGGCCTCGGGAAGTCTAT-3′ and 5′-AGAGCTAGCAGGGCATGCCGCT-3′), 350 uM of each deoxynucleotide triphosphate (dNTP) and 0.75 U Expand Long Template PCR enzyme (Boehringer-Mannheim, Indianapolis, IN). Reactions were carried out under cycle parameters of 1 cycle of 94°C for 7 min, followed by 30 cycles of 1 min at 94°C and 6 min at 68°C, followed by a final extension of 10 min at 68°C. All PCR reactions were hot-started by adding the dNTPs separately during the first cycle of 94°C. Amplified prod-

*Correspondence to: National Institute of Environmental Health Sciences, MD A3-05, PO Box 12233, Research Triangle Park, NC 27709, USA. Fax: +919-541-5472. E-mail: taylor@niehs.nih.gov

Received 10 December 2001; Revised 2 April 2002; Accepted 4 April 2002

DOI 10.1002/ijc.10497

ucts were separated by electrophoresis in a 1% agarose gel 40 cm in length using TBE buffer (45 mM Tris-borate, pH 8.3 and 2 mM EDTA) (Fig. 1). The molecular size marker VII (Roche Applied Science, Indianapolis, IN) and a 123 bp ladder (Invitrogen, Carlsbad, CA) were run in approximately every 7th and 8th lane on all gels to minimize artifacts due to gel distortion. Gels were stained with ethidium bromide and destained in water to allow direct visualization of the alleles. PCR products were first run to determine approximate allele sizes, then were re-run together in groups of similar size for longer times to achieve more refined sizing of specific alleles. When the preliminary allele length typing was complete, all alleles of each length were run again and compared to ensure consistency of length assignments across the entire data set. The genotyping was carried out in a blinded fashion as to case-control status.

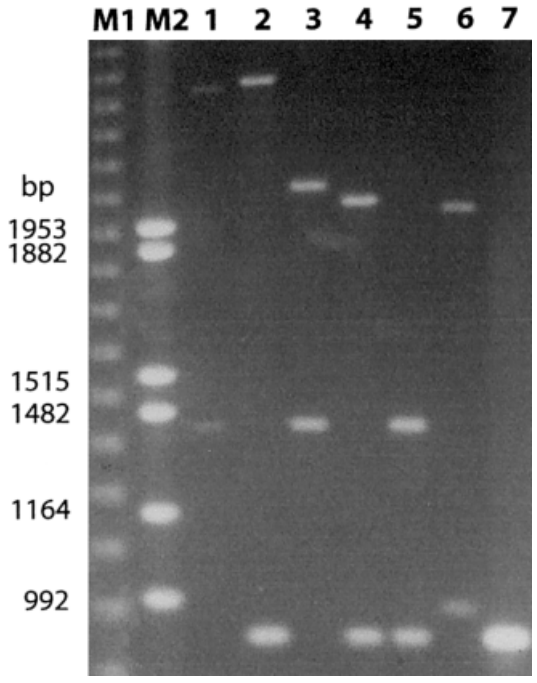

FIGURE 1 – PCR amplification of the HRAS VNTR. PCR amplification of the HRAS minisatellite was carried out as described in the Material and Methods. PCR products were separated in 40 cm long, 1% agarose gels. The markers used were a 123 bp ladder (Invitrogen) (M1) and marker VII (Roche Applied Science) (M2); fragment sizes are indicated for the marker VII. HRAS alleles identified were: sample 1, a2/a4; sample 2, a1/a4+1; sample 3, a2/a3+4; sample 4, a1/a3+2; sample 5, a1/a2; sample 6, a1+2/a3; sample 7, a1/a1.

To assess the robustness of the PCR assay, we compared PCR-based HRAS allelotyping on 355 subjects for whom Southern blotting for HRAS had already been carried out (unpublished). PCR detected all alleles found by Southern blotting, including the largest alleles in heterozygous subjects. In no instance did PCR fail to detect an allele identified by Southern blotting and in fact, PCR provided much greater resolution, allowing us to discriminate between alleles that differed by a single repeat. PCR has been used in a number of publications for HRAS allelotyping.[9,14]

The nomenclature and categorization of genotypes has been described previously.[9,15–18] The 4 most common alleles are labeled as a1, a2, a3 and a4. The other alleles are labeled in terms of the difference in number of 28 bp repeat units from that of the common allele closest in size. For example 'a1+1' refers to the allele with 1 28 bp repeat unit more than a1, 'a1-1' refers to the allele with 1 28 bp repeat unit less than a1. They are referred to as 'rare alleles'.

Statistical analyses

Distributions of demographic characteristics and smoking history were compared between cases and controls and differences tested by χ^2 tests (2-sided). The same was done for distributions of *HRAS1* genotypes. Genotype data for blacks and whites were analyzed separately. Three persons (1 case, 2 controls) who classified themselves as other than black or white were excluded from analysis.

TABLE I – DISTRIBUTION OF BLADDER CANCER RISK FACTORS BY CASES AND CONTROLS

Risk factor	Cases (%) $n = 221$	Controls (%) $n = 202$	p-value[1]
Age at interview, years			
≤60	56 (30)	61 (25)	
61–65	42 (24)	49 (19)	
66–70	49 (17)	35 (22)	
> 70	74 (28)	57 (33)	0.22
Gender			
Female	52 (24)	38 (19)	
Male	169 (76)	164 (81)	0.24
Race			
Black	17 (8)	9 (4)	
White	203 (92)	191 (95)	
Other	1 (0)	2 (1)	0.32
Smoking status			
Never	37 (17)	75 (37)	
Quit	126 (57)	106 (52)	
Current	57 (26)	21 (10)	0.001
Number of years smoked			
0	37 (17)	75 (37)	
1–10	12 (5)	20 (10)	
11–30	54 (25)	58 (29)	
> 30	116 (53)	49 (24)	0.001
Number of pack-years smoked			
0	40 (18)	78 (39)	
1–10	18 (8)	20 (10)	
11–35	52 (24)	53 (26)	
>35	110 (50)	50 (25)	0.001

[1]Based on χ^2 test.

The allele frequencies for a1, a2, a3 , a4 and rare alleles and their 95% confidence intervals (CI) were calculated. A χ^2 goodness-of-fit statistic ($df = 1$) was used to test deviance from Hardy-Weinberg equilibrium. For this purpose, observed numbers of individuals with different genotypes were compared to numbers expected under Hardy-Weinberg equilibrium for the given allele frequencies.

Logistic regression models were used to estimate the odds ratios (OR) and 95% CI for the effects of *HRAS1* genotypes on bladder cancer risk. The reference group consisted of subjects with 2 of the common alleles (*i.e.*, a1, a2, a3 or a4). Bladder cancer OR were estimated for subjects with 1 or 2 rare alleles and adjusted for age (as a continuous variable), gender and smoking (current, former or never smoker). The cases in our study varied in the time that elapsed between the diagnosis of the bladder tumor and their inclusion in the study (median is 2.6 years, interquartile range: 0.9–6.8 years). Therefore, we did separate analyses for cases enrolled in the study within 1 year of diagnosis and cases enrolled more than 1 year after diagnosis. We define the first group as 'incident cases,' which means that they are considered as *newly diagnosed* events during the recruitment period for our study. The latter groups is defined as 'prevalent' cases, which means that they were *existing* cases of cancer at the time of recruitment, who had been diagnosed in the past.[19] Prevalent bladder cancer cases on average have a better prognosis than incident cases, because only the cases with a relatively good prognosis will survive long enough to be able to be included in the study several years after diagnosis of the tumor. Among incident and prevalent cases separately, we also examined the distributions of tumor grade, stage and age at diagnosis by *HRAS1* genotype. Differences in distributions were tested by a χ^2 test (tumor grade), Fisher's exact test (tumor stage) and Wilcoxon's rank sum test (age at diagnosis).

RESULTS

Cases and controls were similar with respect to age, gender and ethnicity, on which they were frequency-matched (Table I). Among the cases there were significantly more smokers than among the controls and on average cases had been smoking for a longer period of time than the controls.

Table II shows the complete genotype distributions for cases and controls. The allele frequencies of a1, a2, a3, a4 and rare alleles in cases were 0.59 (95% CI = 0.54–0.64), 0.13 (95% CI = 0.10–0.16), 0.06 (95% CI = 0.04–0.08), 0.03 (95% CI = 0.01–0.05) and 0.19 (95% CI = 0.15–0.23), respectively. In controls, the respective frequencies were 0.62 (95% CI = 0.57–0.66), 0.12 (95% CI = 0.08–0.15), 0.06 (95% CI = 0.04–0.08), 0.03 (95% CI = 0.01–0.05) and 0.18 (95% CI = 0.14–0.22). Genotype distributions in cases and controls were in Hardy-Weinberg equilibrium ($p > 0.4$).

White subjects having 1 rare allele and 1 common allele did not show an increased overall bladder cancer risk relative to those who were homozygous for common alleles (OR = 0.85; 95% CI = 0.53–1.38) (Table III). Having 2 rare alleles was related to a slight, but non-significant increase in overall risk (OR = 1.74; 95% CI = 0.56–5.44). The risk for those having 1 or 2 rare alleles was 0.93 (95% CI = 0.59–1.47).

According to the original description by Krontiris *et al.*,[20] the class of alleles that we label as rare should be subdivided in an 'intermediate' category with an allele frequency of ≥0.5% and a 'rare' category with an allele frequency <0.5%. When we adopt this more strict definition of 'rare' alleles in our analysis, we find that among the cases 88% have no rare alleles, 10% have 1 rare allele and 1% have 2 rare alleles. Among the controls these proportions are virtually the same: 88.5%, 11% and 0.5%, respectively ($p > 0.6$). Thus, adopting this classification does not change our results and therefore, we decided to use the more commonly used dichotomization between 'common' (a1, a2, a3, a4) and 'rare' alleles[9,15–18] in all further analyses.

TABLE II – *HRAS1* GENOTYPE DISTRIBUTIONS FOR CASE SUBJECTS AND CONTROL SUBJECTS

Allele 1	Allele 2	Cases	Controls
Homozygous for common alleles[1]			
a1	a1	83	76
a1	a2	32	28
a1	a3	18	16
a1	a4	6	6
a2	a2	4	2
a2	a3	5	2
a2	a4	3	4
a3	a3	0	2
a3	a4	1	0
a4	a4	0	0
Total		152	136
Heterozygous - 1 common, 1 rare allele[1]			
a1 − 1	a2	1	0
a1	a1 − 1	0	2
a1	a1 + 1	1	6
a1	a1 + 2	1	2
a1	a1 + 4	6	8
a1	a1 + 6	1	2
a1	a1 + 9	0	2
a1	a1 + 15	2	2
a1	a2 + 1	3	0
a1	a2 + 13	1	0
a1	a2 + 14	1	0
a1	a2 + 21	0	1
a1	a2 + 22	2	4
a1	a3 + 1	4	3
a1	a3 + 2	2	1
a1	a3 + 3	1	1
a1	a3 + 4	1	1
a1	a3 + 15	1	1
a1	a3 + 16	0	1
a1	a3 + 17	3	3
a1	a4 + 1	6	4
a1	a4 + 2	1	0
a1 + 1	a2	1	2
a1 + 1	a4	0	1
a1 + 2	a3	1	0
a1 + 4	a2	0	1
a1 + 4	a3	1	0
a1 + 4	a4	0	1
a1 + 6	a3	1	0
a2	a1 + 4	1	0
a2	a1 + 10	0	1
a2	a1 + 13	0	1
a2	a2 + 1	0	1
a2	a3 + 1	1	0
a2	a3 + 4	1	0
a2	a3 + 12	1	0
a2	a3 + 16	2	0
a2	a3 + 17	1	1
a2	a4 + 1	2	2
a2 + 1	a3	0	1
a2 + 3	a3	0	1
a3	a3 + 1	0	1
a3	a4 + 1	1	0
a3 + 3	a4	1	0
Total		53	58
Homozygous for rare allele[1]			
a1 − 2	a1 − 2	1	0
a1 − 2	a1 + 2	0	1
a1 + 1	a2 + 1	0	1
a1 + 1	a3 + 1	1	0
a1 + 1	a3 + 16	1	0
a1 + 4	a1 + 4	1	0
a1 + 4	a1 + 12	0	1
a1 + 4	a2 + 20	1	0
a1 + 6	a1 + 6	1	0
a1 + 6	a2 + 9	1	0
a1 + 6	a4 + 1	1	0
a1 + 13	a3 + 17	0	1
a2 + 1	a3 + 17	1	0
a2 + 1	a4 + 1	1	0
a2 + 13	a3 + 17	2	0
a3 + 1	a3 + 1	0	1
a3 + 1	a3 + 5	0	1
a3 + 1	a3 + 17	1	0
a3 + 2	a3 + 2	1	0
a3 + 17	a3 + 14	1	0
Total		15	6

[1]a1, a2, a3 and a4 are the common alleles. The rare allele labels are based on the difference in number of base pair repeat units from that of the common allele closest in size.

TABLE III – ASSOCIATIONS OF *HRAS1* GENOTYPE WITH BLADDER CANCER, STRATIFIED BY ETHNICITY, AND SEPARATELY FOR INCIDENT AND PREVALENT CASES

HRAS1 genotype	Controls n (%)	All cases *n* (%)	OR[1]	95% CI	Incident cases *n* (%)	OR[1] incident	95% CI	Prevalent cases *n* (%)	OR[1] prevalent	95% CI
White subjects	$n = 191$	$n = 203$			$n = 53$			$n = 150$		
2 common alleles	132 (69)	146 (72)	1		34 (64)	1		112 (75)	1	
1 common/1 rare allele	53 (28)	48 (24)	0.85	(0.53–1.38)	15 (28)	1.17	(0.57–2.37)	33 (22)	0.77	(0.45–1.31)
2 rare alleles	6 (3)	9 (4)	1.74	(0.56–5.44)	4 (8)	3.21	(0.75–13.72)	5 (3)	1.33	(0.37–4.85)
1 or 2 rare alleles	59 (31)	57 (28)	0.93	(0.59–1.47)	19 (36)	1.34	(0.69–2.61)	38 (25)	0.82	(0.49–1.36)
Black subjects	$n = 9$	$n = 17$			$n = 4$			$n = 13$		
2 common alleles	4 (44)	6 (35)			1 (25)			5 (38)		
1 common/1 rare allele	5 (56)	5 (29)			1 (25)			4 (31)		
2 rare alleles	0 (0)	6 (35)			2 (50)			4 (31)		
1 or 2 rare alleles	5 (56)	11 (65)								

[1]Adjusted for age (continuous), gender and smoking status (current, former, never). CI, confidence interval.

The frequency of rare alleles was higher in black cases and controls than among whites. In black subjects, the combination of 2 rare alleles was observed in approximately one-third (6 of 17) of the cases, but in none of the 9 controls, suggesting increased risk from this genotype *vs.* the genotype with 0 or 1 rare allele ($p = 0.06$). The number of black subjects in our study was too small to be able to estimate meaningful odds ratios. Because of the possibility of effect-modification by ethnicity, however, we restricted all further analyses to whites only.

Among incident cases the proportion of subjects with 1 or 2 rare alleles was higher than among prevalent cases (Table III: 36% *vs.* 25%, respectively), although this difference was not statistically significant ($p = 0.14$). Limiting the analysis to incident cases, we observed a non-significant increase in risk for subjects having 2 rare *HRAS1* alleles (OR = 3.21; 95% CI = 0.75–13.72). When the analysis was limited to prevalent cases the OR associated with 2 rare alleles was 1.33 (95% CI = 0.37–4.85). Having 1 rare allele was not clearly associated with risk in either group.

Incident cases with 1 or 2 rare alleles ($n = 19$) had a higher proportion of Grade IV tumors than patients without any rare alleles ($n = 34$) (47% *vs.* 27%, respectively), although the difference was not statistically significant ($p = 0.33$). Stage information was available for 19 incident cases. Those with 1 or 2 rare alleles ($n = 6$) had more often a higher stage tumor (T2/B1/P2 or higher) than patients without any rare alleles ($n = 13$) (83% *vs.* 38%, respectively), but this difference was not statistically significant either ($p = 0.14$). There was no substantial difference in median age at diagnosis between those with 1 or 2 rare alleles and those without any rare alleles (64 years *vs.* 66 years, respectively) ($p = 0.68$). Among prevalent cases there were no differences between those with 1 or 2 rare alleles and those without any rare alleles for either grade, stage or age at diagnosis.

DISCUSSION

Some earlier studies reported a 2–3-fold increased bladder cancer risk from rare *HRAS1* VNTR alleles.[1,11] These estimates were based on allele frequencies and on the proportion of subjects with 1 or more rare alleles among bladder cancer cases and controls. We find little evidence that rare alleles are associated with overall bladder cancer risk at that level, although our study had a power of more than 80% to detect a risk estimate of this size, if it existed (2-sided $\alpha = 0.05$). Our study can not exclude the hypothesis that rare alleles may be associated with a subset of tumors with poor prognosis. Like us, Bittard *et al.*[12] found no evidence for a relationship between rare *HRAS1* alleles and bladder cancer risk, but they showed that rare alleles were associated with large tumor size, aneuploidy and vascular invasion (all statistically significant) and with shorter disease-free survival (non-significant).

One of the strengths of our study is the use of a PCR-based long-gel electrophoretic assay, with which we achieved a single VNTR repeat resolution. Previous studies used Southern-blot based assays, which have a lower resolution than PCR-based methods and therefore a lower sensitivity to detect rare alleles.[18] A meta-analysis by Krontiris *et al.*,[1] showed that in studies that used Southern-blot based analysis the frequency of rare alleles was 9%. Recent studies on non-Hodgkin lymphoma, lung, breast and ovarian cancer using PCR-based methodologies, report rare allele frequencies ranging from 13–17% in the control population[15–18] which is similar to our results. In 2 of these studies fluorescent primers and size fractionations were used and detection was carried out on an automated sequencer.[17,18] These techniques may be even more accurate in detecting rare alleles than the PCR-based long-gel electrophoretic assay. The distribution of common and rare alleles in our control population, however, was not different from theirs.

Some studies using PCR-based methods observed fewer a3 and a4 common alleles, but a greater number of rare alleles close in size to a3 and a4 than Southern-blot based methods.[17] Preferential amplification of shorter alleles has been a concern with PCR-based methods.[21] If such a problem existed, an increased number of the short a1 and a2 alleles would be expected and not an excess of the long rare alleles in the a3 and a4 size range.

According to the original Krontiris description the class of alleles that we label as 'rare' should be subdivided in an 'intermediate' category with an allele frequency ≥ 0.5 and a 'rare' category with an allele frequency < 0.5.[4,20] In one of his early studies on this subject, where he used this strict definition, rare alleles could only be detected in cancer patients and not in healthy controls.[20] Until now only very few studies have adopted this classification. In some of them this led to greater effects of the rare alleles (on breast cancer,[4] lung cancer in blacks,[22] colorectal cancer[23]), but in others it did not (ovarian cancer[24]). When we adopted this classification, genotype distributions did not differ between cases and controls. Thus, using this more strict definition of 'rare' alleles did not change our conclusions. We must note however, that because only very few people in our population had 2 rare alleles according to Krontiris' definition, it is very hard to study them as a separate group. This is probably also the reason that most other studies used the 'common' (a1, a2, a3, a4) *vs.* 'rare' (all other alleles) definition, as we did here.

Our use of a hospital-based control population could have led to a bias toward the null, if the presence of rare alleles were related to the diagnoses presenting in the control population (the most common being benign prostatic hypertrophy and impotence). As mentioned previously, however, the allele distribution in our hospital-based control population was similar to that of population-based control groups in studies that made use of PCR-based allelotyping methods.[17] The higher frequency of rare alleles in black compared to white subjects has been reported previously for

other cancers[4,25] as has stronger association in blacks between rare *HRAS1* alleles and cancer risk.[4]

Although we did not find evidence that *HRAS1* genotype is an important determinant of bladder cancer risk by itself, there is a possibility that it affects bladder cancer risk in combination with other genes (gene–gene interaction). Phelan *et al.*[26] for example, showed that the risk for ovarian cancer among women carrying both BRCA1 mutations and rare HRAS1 alleles was stronger than what would be expected on the basis of the separate gene effects.

We caution that the number of incident cases in our study is too small to rule out a possible association between the genotype and a subgroup of tumors with a poor prognosis. Such an association should be re-examined with a larger number of subjects, making use of the current precise allele-sizing methods. In addition, it would be important to collect not only information on stage and grade, that we present here, but also detailed information on therapy and survival. If the HRAS1 genotype affects survival rates, or is even associated with differential treatment effects, this would lend strong evidence to the hypothesis of an association between HRAS1 genotype and a subgroup of bladder tumors with a poor prognosis.

Future studies should either be restricted to incident cases or the analyses should be stratified by incident and prevalent cases. This issue is often disregarded in studies of genetic susceptibility because genotype does not change with time, but stratification is required if genotype is associated with prognosis or survival. Although there is some evidence that the *HRAS1* VNTR binds members of the rel/NF-κB transcriptional regulatory system and may affect transcription of *HRAS1*,[7] there is no evidence to date that the classification into 'common' and 'rare' alleles has any functional significance. Until biologic function can be ascribed to the *HRAS1* VNTR and then to individual alleles, the importance of this VNTR as a determinant of cancer risk is likely to remain intriguing but uncertain.

ACKNOWLEDGEMENTS

The authors thank P. Blanton and L. Lansdell for data handling, sample retrieval and management, Dr. M. Stern for helpful discussions and Drs. G. Cooper and J. Schroeder for critical review of the manuscript. Dr. C. van Gils is supported through a fellowship of the Dutch Cancer Society.

REFERENCES

1. Krontiris TG, Devlin B, Karp DD, Robert NJ, Risch N. An association between the risk of cancer and mutations in the *HRAS1* minisatellite locus. N Engl J Med 1993;329:517–23.
2. Capon DJ, Chen EY, Levinson AD, Seeburg PH, Goeddel DV. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 1983;302:33–7.
3. Krontiris TG, DiMartino NA, Colb M, Parkinson DR. Unique allelic restriction fragments of the human Ha-ras locus in leukocyte and tumour DNAs of cancer patients. Nature 1985;313:369–74.
4. Garrett PA, Hulka BS, Kim YL, Farber RA. HRAS protooncogene polymorphism and breast cancer. Cancer Epidemiol Biomarkers Prev 1993;2:131–8.
5. Kasperczyk A, DiMartino NA, Krontiris TG. Minisatellite allele diversification: the origin of rare alleles at the *HRAS1* locus. Am J Hum Genet 1990;47:854–9.
6. Conway K, Edmiston SN, Hulka BS, Garrett PA, Liu ET. Internal sequence variations in the Ha-ras variable number tandem repeat rare and common alleles identified by minisatellite variant repeat polymerase chain reaction. Cancer Res 1996;56:4773–7.
7. Trepicchio WL, Krontiris TG. Members of the rel/NF-κB family of transcriptional regulatory proteins bind the *HRAS1* minisatellite DNA sequence. Nucleic Acids Res 1992;20:2427–34.
8. Green M, Krontiris TG. Allelic variation of reporter gene activation by the *HRAS1* minisatellite. Genomics 1993;17:429–34.
9. Lindstedt BA, Ryberg D, Haugen A. Rare alleles at different *VNTR* loci among lung-cancer patients with microsatellite instability in tumors. Int J Cancer 1997;70:412–5.
10. Conway K, Edmiston S, Fried DB, Hulka BS, Garrett PA, Liu ET. Ha-ras rare alleles in breast cancer susceptibility. Breast Cancer Res Treat 1995;35:97–104.
11. Hayward NK, Keegan R, Nancarrow DJ, Little MH, Smith PJ, Gardiner RA, Seymour GJ, Kidson C, Lavin MF. c-Ha-ras-1 alleles in bladder cancer, Wilms' tumour and malignant melanoma. Hum Genet 1988;78:115–20.
12. Bittard H, Descotes F, Billerey C, Lamy B, Adessi GR. A genotype study of the c-Ha-ras-1 locus in human bladder tumors. J Urol 1996;155:1083–8.
13. Bell DA, Taylor JA, Paulson DF, Robertson CN, Mohler JL, Lucier GW. Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen-metabolism gene glutathione s-transferase M1 (*GSTM1*) that increases susceptibility to bladder cancer. J Natl Cancer Inst 1993;85:1159–64.
14. Ding S, Larson GP, Foldenauer K, Zhang G, Krontiris TG. Distinct mutation patterns of breast cancer-associated alleles of the *HRAS1* minisatellite locus. Hum Mol Genet 1999;8:515–21.
15. Calvo R, Pifarre A, Rosell R, Sanchez JJ, Monzo M, Ribera JM, Feliu E. H-RAS 1 minisatellite rare alleles: a genetic susceptibility and prognostic factor for non-Hodgkin's lymphoma. J Natl Cancer Inst 1998;90:1095–8.
16. Rosell R, Calvo R, Sanchez JJ, Maurel J, Guillot M, Monzo M, Nunez L, Barnadas A. Genetic susceptibility associated with rare *HRAS1* variable number of tandem repeats alleles in Spanish non-small cell lung cancer patients. Clin Cancer Res 1999;5:1849–54.
17. Firgaira FA, Seshadri R, McEvoy CR, Dite GS, Giles GG, McCredie MR, Southey MC, Venter DJ, Hopper JL. *HRAS1* rare minisatellite alleles and breast cancer in Australian women under age forty years. J Natl Cancer Inst 1999;91:2107–11.
18. Weitzel JN, Ding S, Larson GP, Nelson RA, Goodman A, Grendys EC, Ball HG, Krontiris TG. The *HRAS1* minisatellite locus and risk of ovarian cancer. Cancer Res 2000;60:259–61.
19. Rothman KJ, Greenland S. Modern epidemiology. Philadelphia: Lippincott-Raven Publishers, 1998.
20. Krontiris TG, DiMartino NA, Colb M, Mitcheson HD, Parkinson DR. Human restriction fragment length polymorphisms and cancer risk assessment. J Cell Biochem 1986;30:319–29.
21. Krontiris TG. Re: *HRAS1* rare minisatellite alleles and breast cancer in Australian women under age forty years. J Natl Cancer Inst 2000; 92:755A–6A.
22. Sigimura H, Caporaso NE, Modali RV, Hoover RN, Resau JH, Trump BF, Longergan JA, Krontiris TG, Mann DL, Weston A, et al. Association of rare alleles of the Harvey ras protooncogene locus with lung cancer. Cancer Res 1990;50:1857–62.
23. Klingel R, Mittelstaedt P, Dippold WG, Meyer zum Buschenfelde KH. Distribution of Ha-ras alleles in patients with colorectal cancer and Crohn's disease. Gut 1991;32:1508–13.
24. O'Briant K, Chrysson N, Hunter V, Tyson F, Tanner M, Daly L, George SL, Berchuck A, Soper J, Fowler W, et al. Ha-ras polymorphisms in epithelial ovarian cancer. Gynecol Oncol 1992;45:299–302.
25. Devlin B, Krontiris T, Risch N. Population genetics of the *HRAS1* minisatellite locus. Am J Hum Genet 1993;53:1298–305.
26. Phelan CM, Rebbeck TR, Weber BL, Devilee P, Ruttledge MH, Lynch HT, Lenoir GM, Stratton MR, Easton DF, Ponder BA, Cannon-Albright L, Larsson C, Goldgar DE, Narod SA. Ovarian cancer risk in *BRCA1* carriers is modified by the *HRAS1* variable number of tandem repeat (*VNTR*) locus. Nat Genet 1996;12:309–11.